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Cell Biology

ATCC® Number:	**CRL-10463™** Order this item	**Price:**	**$330.00**
Designations:	A-HER2 [4D5; NB9644P28]	**Depositors:**	Genentech, Inc.
Isotype:	mouse IgG1		
Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	suspension
Organism:	*Mus musculus* (B cell); *Mus musculus* (myeloma) (mouse (B cell); mouse (myeloma))	**Morphology:**	lymphoblast
Source:	**Organ:** spleen **Cell type: hybridoma:** B lymphocyte;		
Cellular Products:	immunoglobulin; monoclonal antibody; against HER2 receptor		
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Related Cell Culture Products

Comments:	Animals were immunized with HER2-amplified NIH 3T3 transformed cells. Spleen cells were fused with P3X63Ag8.653 myeloma cells. The antibody binds to the extracellular domain of the HER2 receptor and inhibits the growth of SK-BR-3 (ATCC HTB-30) breast tumor cells. [30816] The SK-BR-3 cell line overexpresses the HER2/c-erb-2 gene product. [49665] The antibody prevents HER2/c-erb-2 transformed NIH 3T3 cells from forming colonies in soft agar. [49665] It does not cross-react with the human epidermal growth factor (EGF) receptor and it will immunoprecipiate p185HER2. [49662] [49665]
Propagation:	**ATCC complete growth medium:** Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%; fetal bovine serum, 10%

	Temperature: 37.0C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%
Subculturing:	**Protocol:** Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 X 10(5) viable cells/ml. **Interval:** Maintain cell density between 5 X 10(4) and 1 X 10(6) viable cells/ml. **Medium renewal:** Add fresh medium every 2 to 3 days (depending on cell density)
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2002 recommended serum: ATCC 30-2020
References:	30816: Hudziak RM , et al. Monoclonal antibodies directed to the Her2 receptor. US Patent 5,677,171 dated Oct 14 1997 32176: Hudziak RM , et al. In vivo tumor detection assay. US Patent 5,720,937 dated Feb 24 1998 49662: Fendly BM , et al. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 50: 1550-1558, 1990. PubMed: 1689212 49665: Hudziak RM , et al. p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol. 9: 1165-1172, 1989. PubMed: 2566907 88867: Baughman SA , Shak S . Dosages for treatment with anti-Erb2 antibodies. US Patent 6,627,196 dated Sep 30 2003 90263: Carter PJ , Presta LG . Method for making humanized antibodies. U.S. Patent 6,800,738 dated Oct 5 2004 90264: Carter PJ , Presta LG . Method for making humanized antibodies . U.S. Patent 6,719,971 dated Apr 13 2004

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Cell Biology

ATCC® Number:	**HB-8696™** Order this item	**Price:**	**$330.00**
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Isotype:	IgG1		
Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	suspension
Organism:	*Mus musculus* (B cell); *Mus musculus* (myeloma) (mouse (B cell); mouse (myeloma))	**Morphology:**	lymphoblast
Source:	**Cell type: hybridoma:** B lymphocyte;		
Cellular Products:	immunoglobulin; monoclonal antibody; against human breast cancer cells		
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Related Cell Culture Products

Tumorigenic:	Yes, forms ascites in BALB/c mice
Comments:	Animals were immunized either with live cells or with membrane extracts from human breast cancer cell lines. Spleen cells were fused with Sp2/0-Ag14 myeloma cells. By immunofluorescence, the antibody is specific for neoplastic breast tissue with some weak cross-reaction with other neoplasms. The antibody reacts with a 210000 dalton protein found in cancerous human breast tissue.
Propagation:	**ATCC complete growth medium:** Modified Dulbecco's medium, 80%; fetal bovine serum, 20%
Subculturing:	Cultures can be maintained by addition or replacement of fresh medium. Start cultures at

	2 X 10 exp5 cells/ml and maintain between 1 X 10 exp5 and 1 X 10 exp6 cells/ml. **Medium renewal:** Every 2 to 3 days
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 46-X
References:	3895: Frankel AE , et al. Monoclonal anti-human breast cancer antibodies. US Patent 4,753,894 dated Jun 28 1988 70147: Tempest PR , et al. Humanized antibodies to Fc receptors for immunoglobulin G on human mononuclear phagocytes. US Patent 6,500,931 dated Dec 31 2002

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The purpose of this guide is to assist you in the proper administration of Herceptin.

Related Materials

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INDICATION

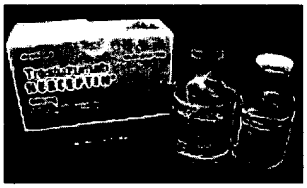
Herceptin® (Trastuzumab) is the only FDA-approved therapeutic for HER2 protein overexpressing metastatic breast cancer. Approved for first-line use in combination with paclitaxel.(1)

Herceptin as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease.(1)

Herceptin is administered weekly and may be given in an outpatient setting.

The HER2 growth factor receptor is rapidly and continuously manufactured. The consistent presence and action of Herceptin may provide ongoing suppression of the receptor activity.(2)

Package Contents(1)



- One preservative-free 440 mg vial of Herceptin (lyophilized).
- One 20 mL vial of Bacteriostatic Water For Injection (BWFI), USP (1.1% benzyl alcohol preserved).

RECONSTITUTION

- Using aseptic technique, **reconstitute with the 20 mL of BWFI**; this yields a multi-dose solution containing 21 mg/mL Herceptin® (Trastuzumab) at a pH of approximately 6
- Keep the multi-dose solution refrigerated at 2°- 8°C (36°- 46°F). When reconstituted with BWFI, the multi-dose solution may be used for 28 days when stored at 2°- 8°C (36°- 46°F). **DO NOT FREEZE OR SHAKE RECONSTITUTED SOLUTION.**
- If the patient has known hypersensitivity to benzyl alcohol, Herceptin must be reconstituted with SWFI (Sterile Water for Injection) (see PRECAUTIONS in full Prescribing Information).

HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

DOSAGE GUIDELINES

- Determine number of mg Herceptin needed based on patient body weight in kilograms.
  - Convert patient weight into kilograms: 2.2 pounds = 1 kilogram:

$$\frac{\text{patient weight in pounds}}{2.2} = \text{patient weight in kilograms}$$

  - Insert patient weight in kilograms into the dosage equation, using either 4 mg (loading dose: 4 mg Herceptin/kg body weight) or 2 mg (maintenance dose: 2 mg Herceptin/kg body weight) as the multiplier:

$$\frac{\text{patient weight in kg} \times \text{multiplier (2 or 4 mg/kg)}}{\text{21 mg/mL}} = \frac{\text{\#\_\_ mL reconstituted Herceptin solution}}{}$$

- Add calculated amount of reconstituted Herceptin solution to 250 mL of 0.9% sodium chloride, USP. DO NOT USE DEXTROSE (5%) SOLUTION.

When reconstituted with BWFI, USP (1.1% benzyl alcohol preserved), the multi-dose Herceptin solution may be used for 28 days, when stored refrigerated at 2°-8°C (36°-46°F). DO NOT FREEZE OR SHAKE RECONSTITUTED SOLUTION.

ADMINISTRATION

- 4 mg/kg loading dose administered over a 90-minute infusion.
- 2 mg/kg maintenance dose can be administered as a 30-minute infusion if the initial loading dose was well tolerated.
- DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.
- Herceptin SHOULD NOT BE MIXED OR DILUTED WITH OTHER DRUGS.

In clinical studies, infusion-associated symptoms were managed with acetaminophen, diphenhydramine, and/or meperidine.

Please see full Prescribing Information, including BOXED WARNINGS.

References:

1. Herceptin® (Trastuzumab) full Prescribing Information; February 2005.

2. Sliwkowski MX, Lofgren JA, Lewis GD, et al. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol. 1999;26(suppl 12):60-70.

Genentech BIOONCOLOGY

Polymorphonuclear Granulocytes Induce Antibody-Dependent Apoptosis in Human Breast Cancer Cells[1]

Bernhard Stockmeyer,[2]* Thomas Beyer,* Winfried Neuhuber,† Roland Repp,* Joachim R. Kalden,* Thomas Valerius,* and Martin Herrmann*

Recent studies in HER-2/neu-targeted immunotherapy demonstrated that polymorphonuclear neutrophils (PMN) mediated Ab-dependent cellular cytotoxicity against HER-2/neu-positive breast cancer cell lines. However, the mechanism of cell death remained unclear. We used several assays to analyze the induction of apoptosis in the breast cancer cell line SK-BR-3 via PMN-dependent Ab-dependent cellular cytotoxicity. In the presence of the HER-2/neu Ab 520C9 and PMN from healthy donors, apoptosis occurred as detected by annexin V binding and disappearance of euploid SK-BR-3 nuclei, which can be differentiated from PMN nuclei by their increased DNA contents. Apoptosis induction was observed with E:T cell ratios as low as 10:1. Laser scanning fluorescence microscopy of TUNEL tumor cells or staining for cleaved cytokeratin-18 further confirmed apoptosis of the SK-BR-3 breast cancer cells. Killing via 520C9 was dependent on the interaction with FcR on PMN, because 1) $F(ab')_2$ fragments of 520C9 mediated no cytotoxicity, 2) target cell death was influenced by a biallelic polymorphism of FcγRIIa on the effector cells, and 3) a bispecific Ab against HER-2/neu and the IgA receptor (FcαRI) expressed on effector cells significantly induced apoptosis. Thus, PMN induce Ab-dependent apoptosis against human breast cancer cells targeted with HER-2/neu-directed mAbs or FcR directed bispecific Abs. ***The Journal of Immunology,*** **2003, 171: 5124–5129.**

Overexpression of the protooncogene HER-2/neu is observed in approximately one-third of breast cancers and a varying proportion of ovarian, gastric, and other epithelial tumors. HER-2/neu expression is an independent risk factor for early relapse and death in breast cancer (1). The combination of the mAb trastuzumab (Herceptin) directed against HER-2/neu and chemotherapy improved the clinical outcome for patients with HER-2/neu-overexpressing metastatic breast cancers (2). HER-2/neu forms heterodimers with other members of the epidermal growth factor receptor family, e.g., epidermal growth factor receptor, HER-3 or HER-4 (3). HER-2/neu is a tyrosine kinase signaling molecule and its overexpression results in constitutive and coreceptor-independent kinase activation. We have previously demonstrated that isolated polymorphonuclear neutrophils (PMN)[3] are potent effector cells against a wide range of malignancies in vitro (4–6). In contrast to T cells, the cytotoxicity of PMN is dependent on the presence of Abs binding to the target cells. Usually, PMN-mediated Ab-dependent cellular cytotoxicity (ADCC) is measured using 3-h ^{51}Cr release assays (7, 8) with high E:T cell ratios up to 1000:1 (9). To recruit cell-mediated effector mechanisms, Abs must interact with Ig FcRs, which are classified as Fcγ-, Fcα-, or Fcε-receptors depending on their specificity for IgG, IgA, or IgE, respectively (10). FcγR are grouped according to their affinity into low affinity receptors—named FcγRII (CD32) and FcγRIII (CD16)—and the high affinity FcγRI (CD64) (11). In vivo FcR-bearing cells like NK cells, monocytes, or PMN are able to infiltrate tumors and to cross-link mAb bound to the surface of tumor cells (12). The myeloid form of FcγRII, e.g., the FcγRIIa expressed on PMN, contains an immunoreceptor tyrosine-based activation motif in its cytoplasmic domain, whereas the FcγRIIb isoforms, e.g., expressed on B cells display immunoreceptor tyrosine-based inhibitory motifs. A genetic polymorphism in the extracellular ligand binding domain of FcγRIIa determines reactivity with human IgG2, murine IgG1, and rat IgG2b isotypes (11). The two alleles differ in a single amino acid at position 131. The arginine-containing allotype (R131) displays an increased affinity for murine IgG1, when compared to the allotype with histidine in this position (H131). Binding of ligands to FcαRI (CD89), FcγRI, and FcγRIIIa initiates intracellular signaling by the FcR common γ-chain, containing immunoreceptor tyrosine-based activation motif. We have recently demonstrated that FcαRI triggers PMN-mediated cytotoxicity even better than the established activating molecules FcγRI and FcγRIIa (13). The pivotal role of FcR for the tumoricidal activities of mAbs has recently been demonstrated in knock-out mice, in which the signaling machinery of FcR was genetically disrupted (14). Recent studies in lymphoma patients showed an association between the FcγRIIIA isotype and the clinical and molecular responses to CD20-directed therapy with mAbs (15).

The capacity to mediate ADCC has been demonstrated in vitro for monocytes/macrophages, NK cells, as well as eosinophilic and neutrophilic granulocytes. PMN are increasingly recognized as an important effector cell population for rejection of malignant tumors in vivo (12, 16, 17). Furthermore, PMN were the predominant effector cell population for the killing of breast cancer cells in the presence of HER-2/neu Abs in vitro (4). However, the mechanism of target cell death remains elusive. Recent therapeutic advances in Ab-mediated HER-2/neu directed cancer therapy have renewed the interest in its mechanism (2).

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; ADCC, Ab-dependent cellular cytotoxicity; PI, propidium iodide; ADAC, Ab-dependent apoptosis of target cells; FSc-SSc, forward scatter vs side scatter.

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Apoptotic cell death is characterized by a breakdown of the membrane lipid asymmetry, by the loss of matrix adhesion and mitochondrial membrane potential, by "boiling" of cytoplasm, condensation of chromatin, and finally by internucleosomal cleavage of the nuclear DNA (18). Exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane already occurs at an early stage of apoptosis and is detected by the binding of annexin V. In late stages, DNA fragmentation and loss of chromatin result in sub-G_1 DNA content of the dying cells. The latter can be detected by propidium iodide (PI) staining in the presence of detergent. In contrast, the conventional short-term ^{51}Cr release assay measures release of cytosolic chromate ions, which can only be observed when the cytoplasmic membrane of the target cells are disrupted in either primary or secondary necrosis. The ^{51}Cr release assay requires high E:T cell ratios, unlikely to occur in vivo. In the present study, we investigated ADCC at low E:T cell ratios. Under these more physiological conditions, we observed an Ab-dependent apoptosis of target cells (ADAC) depending on the presence of both PMN and tumor-specific Abs.

Materials and Methods

Cells and culture conditions

The human breast cancer cell lines SK-BR-3 and MDA-MB 453 were obtained from the American Type Culture Collection (Manassas, VA). Cells were kept in $RF10^+$ medium consisting of RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 3 mM L-glutamine (all from Life Technologies). Experiments reported in this study were in accordance with the Declaration of Helsinki. After informed consent, 10–20 ml of citrate anticoagulated peripheral blood was drawn from healthy volunteers for effector cell preparation.

Isolation of PMNs and mononuclear cells

Isolated neutrophils were obtained by a method slightly modified from that described in Ref. 19. Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (Seromed, Berlin, Germany), consisting of 70 and 62% Percoll. After centrifugation, neutrophils were collected between the two Percoll layers, and mononuclear cells from the plasma/Percoll interface. Remaining erythrocytes were removed by hypotonic lysis. Purity of neutrophils was determined by cytospin preparations and exceeded 95%, with few contaminating eosinophils and <1% mononuclear cells. Viability of cells tested by trypan blue exclusion was >95%.

mAb and Ab constructs

Murine whole Ab and $F(ab')_2$ against the proto-oncogene product HER-2/neu was 520C9 (mIgG1; Medarex, Annandale, NJ) (20). Control Ab was Th 69 (mIgG1) against CD7 (21). FITC-labeled $F(ab')_2$ of Ab to mouse IgG were from Cappel (Cochranville, PA).

FcR Ab A77 to FcαRI (mIgG1, CD89) (22) was kindly provided by Medarex. mAb AT10 (mIgG1) (23) and mAb 41H16 (mIgG2a) (24), both against FcγRII, were generous gifts from M. Glennie (Tenovus Research Laboratory, Southampton, U.K.) and B. Longenecker (University of Alberta, Edmonton, Alberta, Canada), respectively.

Bispecific Ab (FcαRI × HER-2/neu) was a generous gift from M. Glennie (Tenovus Research Laboratory, Southampton, U.K.). The bispecific Ab was produced by chemically cross-linking F(ab') of mAb 520C9 (HER-2/neu), and A77 (FcαRI; CD89), as described elsewhere (25). The bispecific Ab showed binding to both E:T cells consistent with the specificity pattern of the parental Abs.

Allotyping for the R-H131 polymorphism of FcγRIIa

Phenotyping for the R131 and H131 alleles of the FcγRIIa was performed by quantitative indirect flow cytometry of peripheral blood monocytes (26). Cells were stained with mAb 41H16, which selectively recognizes the FcγRIIa-R131 alloform, or by mAb AT10 recognizing both FcγRIIa alloforms.

ADCC

^{51}Cr release assays were performed as described elsewhere (27). Briefly, target cells were labeled with 200 μCi ^{51}Cr for 2 h. After extensive washing with $RF10^+$, cells were adjusted to 10^5 per milliliter. PMN at the indicated E:T cell ratio, sensitizing Abs at a final concentration of 2 μg/ml, and $RF10^+$ were added to round-bottom microtiter plates (Nunc, Roskilde, Denmark). Assays were started by adding 50 μl of the target cells, resulting in a final volume of 200 μl. After 3 h at 37°C, assays were stopped by centrifugation, and ^{51}Cr release from triplicates was measured. Percentage of cellular cytotoxicity was calculated using the formula: specific lysis = (experimental cpm − basal cpm/maximal cpm − basal cpm) × 100%.

Maximal ^{51}Cr release was determined by adding perchloric acid to target cells at a final concentration of 3%. Basal release was measured in the absence of sensitizing Abs and effector cells. No Ab-independent cytotoxicity was observed with PMN as effector cells in the absence of targeting Abs.

For analyses of ADAC by flow cytofluorometry, freshly isolated PMN in $RF10^+$ were added to 10^5 SK-BR-3 cells to yield an E:T cell ratio of 10:1 in a final volume of 1 ml. Assays were started by addition of mAb or of bispecific Ab at a final concentration of 2 μg/ml, respectively. Assays were incubated for 18 h at 37°C in a humidified atmosphere containing 5% CO_2.

Immunofluorescence analyses

Phosphatidylserine exposure during apoptosis was detected by FITC-labeled annexin V (Roche, Mannheim, Germany), according to the manufacturer's instruction. Necrotic cells were detected by PI uptake. After a 20-min incubation, annexin V binding was measured with an EPICS Profile flow cytometer (Beckman Coulter, Brea, CA). PMN and target cells were distinguished by forward scatter vs side scatter (FSc-SSc) characteristics. Annexin V-positive and PI-negative cells were considered apoptotic.

Nuclear DNA content was analyzed by PI staining after permeabilization of the cytoplasmic membrane with detergent (28). Two to five volumes of PI-Triton staining solution (0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/ml PI) were added to the cell suspension. The DNA content was measured after an incubation at 4°C in the dark for 24 h. Because the nuclei of the SK-BR-3 target cells were hyperdiploid and displayed different morphology compared to PMN, they could easily be differentiated from the nuclei of the PMN by increased PI staining and by characteristic FSc-SSc.

Fluorescence laser scanning microscopy

The in situ end labeling of DNA strand breaks (TUNEL) generated during apoptosis was performed with the In Situ Cell Death Detection Kit, Fluorescein (Roche), according to the manufacturer's instructions for cytospin preparations. A TUNEL reaction mixture without TdT served as a negative control. After ADCC assay for 18 h at 37°C, PMN and SK-BR-3 cells were centrifuged onto microscope slides. Cells were air-dried and fixed with a solution of 4% paraformaldehyde in PBS at pH 7.4 for 30 min. After flushing with PBS, the slides were incubated in 0.1% Triton X-100 with 0.1% sodium citrate on ice for 2 min. The slides were rinsed twice with PBS and the area around the sample was dried. Fifty microliters of the TUNEL reaction mixture was added and the staining was allowed to proceed for 60 min at 37°C in the dark. The slides were washed three times in PBS and were examined by confocal laser scanning microscopy (Bio-Rad, Munich, Germany).

Statistical analysis

Results are expressed as means ± SEM. The statistical significance was calculated with the Student t test (Excel; Microsoft, Richmond, VA).

Results

High E:T cell ratios are required for lysis of SK-BR-3 cells in ^{51}Cr release assays

HER-2/neu-positive SK-BR-3 breast cancer cells were incubated in the presence of the murine IgG1 mAb 520C9 (2 μg/ml) with increasing PMN to target cell ratios. In 3-h ^{51}Cr release assays, mAb mediated relevant lysis only at E:T ratios above 40:1. Twenty-hour ^{51}Cr release assays gave similar results (Fig. 1). No significant lysis was to be observed in the absence of either PMN or 520C9 (data not shown).

PMN induce Ab-dependent apoptosis of SK-BR-3 cells

The mechanism of Ab-mediated cell death induced by PMN remains unclear. Therefore, we analyzed the induction of apoptosis in breast cancer cells during ADCC. Apoptosis of the SK-BR-3 cells was measured by binding of Annexin V^{FITC} to target cells after a 20-h incubation with PMN isolated from healthy donors

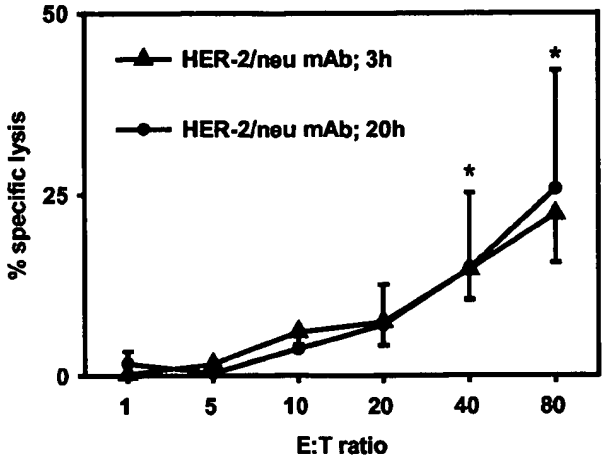

FIGURE 1. High E:T cell ratios are required for lysis of SK-BR-3 cells in Ab-dependent chromium release assays. HER-2/neu-positive SK-BR-3 breast cancer cells were incubated in the presence of the HER-2/neu-directed mAb 520C9 (mouse IgG1) with PMN. Comparing 20-h chromium release assays and 3-h assays, we found no significantly enhanced cellular cytotoxicity in longer term assays. In addition, we observed an increase in the spontaneous release after prolonged assay duration. The E:T ratios are indicated on the ordinate. Conventional mAb mediated significant and relevant ^{51}Cr release (<10% specific lysis) only at E:T ratios ≥40:1. Results from $n \geq 3$ experiments with healthy donors are shown. *, Significant lysis, $p < 0.05$.

(Fig. 2*A*). The assays were run in triplicates and were repeated at least three times. For analysis, cancer cells were gated according to their FSc-SSc characteristics. PI-permeable necrotic cancer cells were excluded from analysis. In the presence of either mAb 520C9 or a bispecific Ab directed against HER-2/neu (expressed by the cancer cells) and FcαRI (CD89) (expressed on the effector cells), PMN significantly triggered apoptosis of cancer cells (Fig. 2*B*). The induction of apoptosis was dependent on the interaction with FcR on effector cells, as $F(ab')_2$ of 520C9 mediated no cytotoxicity. Importantly, we detected significant apoptosis rates already at a low E:T ratio of 10:1.

ADCC results in apoptotic target cells with sub-G_1 DNA contents

Due to their hyperdiploid karyotype, PI-stained G_0/G_1 and G_2 nuclei of SK-BR-3 cells could easily be distinguished from euploid PMN nuclei (Fig. 3*A*). During apoptosis induced by irradiation with UV B, chromatin is degraded and apoptotic cells can be identified by their sub-G_1 DNA content. In contrast, induction of necrosis (e.g., by treatment with methanol, ethanol, or heating for 30 min to 56°C) does not generate nuclei with sub-G_1 DNA content (data not shown). In the presence of conventional HER-2/neu-directed mAb or bispecific Ab (FcαRI × HER-2/neu), SK-BR-3 cells underwent apoptosis, reflected by the disappearance of target cell nuclei with $G_0/_1$-S-G_2 DNA content (Fig. 3*A*). $F(ab')_2$ of HER-2/neu-directed mAb or isotype controls had no effect on SK-BR-3 cell death. The latter results demonstrate the involvement of FcR in the induction of apoptosis. In accordance with our previous data obtained with the chromium release assay (13, 29), targeting FcαRI as cytotoxic trigger molecule on PMN induced significantly more apoptosis than targeting FcγR with conventional mAb (Fig. 3*B*). Similar results were obtained using the HER-/neu positive breast cancer cell line MDA-MB453 (Table I).

In the presence of the murine IgG1 mAb 520C9, PMN of R/R131 individuals were significantly more cytotoxic than those of H/H131 donors

Two allelic forms of FcγRIIa are expressed on myeloid cells. Because chromium release induced by murine IgG1 has been demonstrated to be more efficient with PMN of R/R131 individuals (4), we examined whether this is also true for the induction of apoptosis. ADAC by PMN from three donors homozygous for FcγRIIa-R/R131 or FcγRIIa-H/H131 were compared in the presence of the mAb 520C9. As shown in Fig. 4, the viable nuclei count of the tumor cells, measured by PI staining, was significantly ($p < 0.001$) reduced in the presence of PMN isolated from all donors. However, PMN from R/R131 individuals were significantly more cytotoxic than those from H/H131 donors ($p < 0.001$).

TUNEL confirmed apoptosis of breast cancer cells in ADCC

To further confirm apoptotic cell death of the target cells in ADCC, we used the TUNEL method. PMN and SK-BR-3 cells were coincubated for 20 h in the presence or absence of the mAb 520C9. Subsequently, cells were permeabilized and stained with the TUNEL technique. Nuclear counterstaining was performed with PI. Stained cells were analyzed using laser scanning fluorescence microscopy. In the absence of targeting mAb, we detected only background levels of TUNEL-positive SK-BR-3 nuclei. In contrast, in the presence of the mAb 520C9 we observed numerous apoptotic breast cancer cells (Fig. 2*C*), thus confirming apoptotic death of the tumor cells. PMN could easily be differentiated from the tumor cells by their nuclear morphology. Apoptotic PMN showed bright TUNEL staining, whereas viable PMN were TUNEL-negative. Few contaminating eosinophilic granulocytes showed unspecific dUTP FITC staining (30).

Cytokeratin-18 cleavage confirmed apoptosis of breast cancer cells in ADCC

SK-BR-3 cells were cocultured with PMN at an E:T ratio of 10:1 for 42 h in the presence or absence of HER-2/neu mAb. Adherent cells were stained for a cytoplasmic neoepitope of cytokeratin-18, which is generated by caspase cleavage during late phase of apoptosis of epithelial cells (31). Apoptotic SK-BR-3 cells were almost exclusively observed in the presence of the HER-2/neu mAb. Cytoplasmic staining of apoptotic SK-BR-3 cells could easily be distinguished from rare PMN showing faint, unspecific surface fluorescence (Fig. 2, *D–G*).

Discussion

The aim of this study was to investigate the mechanism of Ab-dependent, PMN-mediated cytotoxicity against breast cancer cells. We used flow cytofluorometry and fluorescence microscopy to investigate changes in target cells indicative for apoptotic vs necrotic cell death. Interestingly, we observed annexin V binding to PI-negative, apoptotic tumor cells during PMN-mediated ADCC. In addition, analyses of cellular DNA content, TUNEL staining, and detection of specific caspase-cleaved cytokeratin-18 fragments confirmed PMN-induced ADAC in breast cancer cells. Interaction between FcR on PMN and target cells was required for the induction of apoptosis. Conventional Abs targeting FcγRII (4), or bispecific Abs targeting FcαRI on PMN initiated apoptosis, whereas isotype controls and $F(ab')_2$ of the HER-2/neu Ab had no apoptosis-inducing effect on breast cancer cells. The $F(ab')_2$ of the mAb 520C9 even increased tumor cell survival, most likely due to stimulation of SK-BR-3 due to cross-linking of HER-2/neu molecules.

The polymorphisms of FcγRIIa (32, 33), FcγRIIb (34), FcγRIIIa (15), and FcγRIIIb (35, 36) have been demonstrated to have clinical implications and functional relevance. In the presence

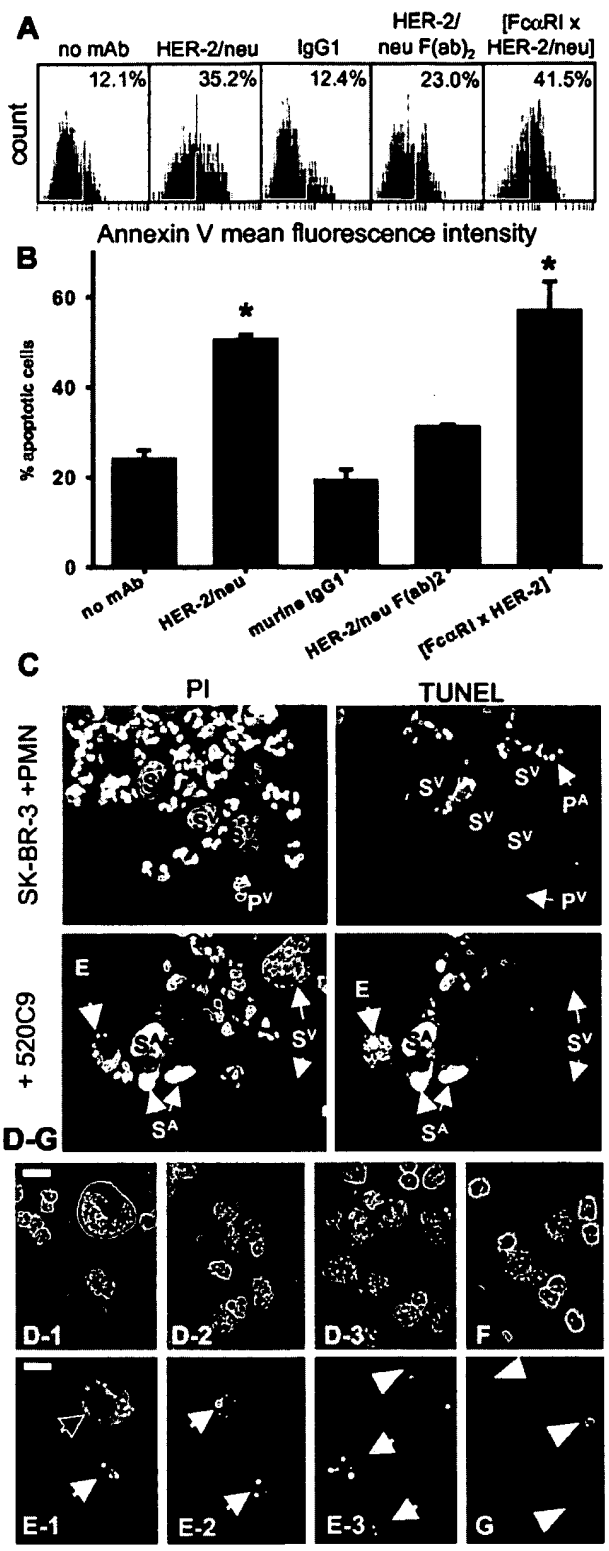

FIGURE 2. Induction of apoptosis in breast cancer cells after coculture with PMN in the presence of HER-2/neu-specific mAb. Annexin V binding of breast cancer cells after coculture with PMN in the presence of HER-2/neu-specific mAb. SK-BR-3 cells and isolated PMN were incubated with the indicated Abs for 20 h. In the presence of the mAb against HER-2/neu or the bispecific Ab targeting HER-2/neu and FcαRI, PMN mediated apoptosis of breast cancer cell line SK-BR-3 (*A*). Killing via 520C9 was specific and dependent on the interaction with FcR on effector cells, as a murine isotype control mAb or $F(ab')_2$ of 520C9 mediated no significant apoptosis, respectively (*B*). One representative of at least three similar experiments is shown; error bars show SD of triplicates of a representative experiment. *, A highly significant increase in apoptotic cells compared to control without mAb, $p < 0.001$. Photomicrographs showing cocultures of PMN and SK-BR-3 stained with the TUNEL technique (*C*). PMN can easily be differentiated from SK-BR-3 cells by their nuclear morphology (*upper left panel*, PI staining). Apoptotic PMN (P^A) show bright TUNEL staining, whereas viable PMN (P^V) are TUNEL-negative (*upper right panel*). In the absence of targeting mAb (*upper panels*), no induction of TUNEL-positive SK-BR-3 nuclei has been observed (S^V). In contrast, in the presence of the mAb 520C9 targeting HER-2/neu on the breast carcinoma cell line SK-BR-3, numerous apoptotic tumor cells (S^A) have been recognized (*bottom right panel*). *E*, A contaminating eosinophilic granulocyte with unspecific dUTP FITC staining (30). PMN-induced cytokeratin-18 cleavage in SK-BR-3 is dependent on the presence of HER-2/neu Abs. SK-BR-3 cells were cocultured with PMN at an E:T ratio of 10:1 for 42 h in the presence (*D*1–3, *E*1–3) or absence (*F* and *G*) of the HER-2/neu mAb 520C9. Adherent cells were stained for a cytoplasmic neoepitope of cytokeratin-18, generated by caspase-mediated cleavage during the late phase of apoptosis of epithelial cells. Apoptotic SK-BR-3 cells (arrows) were almost exclusively observed in the presence of the HER-2/neu mAb (B1–3). Cytoplasmic staining of apoptotic SK-BR-3 cells (arrows) can easily be distinguished from rare unspecific surface binding of PMN (arrowheads). The space bar represents 20 μm.

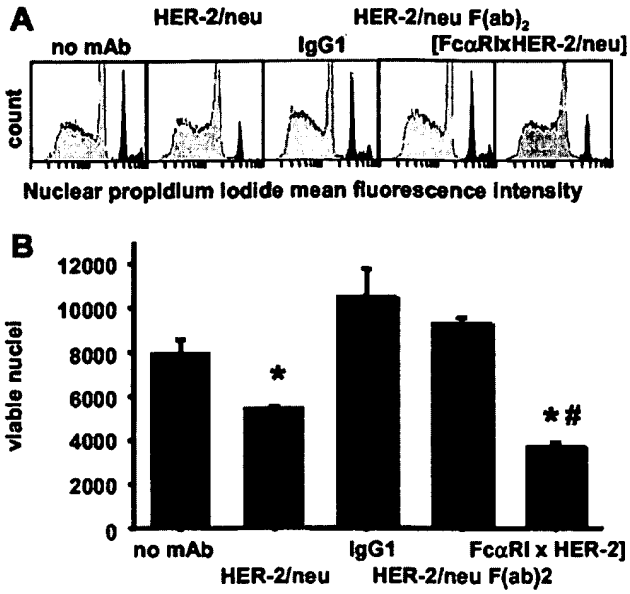

FIGURE 3. Nuclear DNA content of SK-BR-3 after ADAC. Due to their hyperdiploid karyotype, PI-stained G_0/G_1 and G_2 nuclei of SK-BR-3 cells (*A*, black histogram) can easily be differentiated from PMN nuclei (gray histogram). During apoptosis, nuclei are degraded and their DNA content decreases (*A*). In the presence of the conventional HER-2/neu mAb 520C9, or of the bispecific Ab (HER-/neu × FcαRI) SK-BR-3 cells underwent apoptosis. In contrast, $F(ab')_2$ of mAb 520C9 or isotype controls (murine IgG1) did not effect the cell death of SK-BR-3. Targeting of the FcαRI as cytotoxic trigger molecule on PMN induced significantly more lysis than targeting of the FcγR with conventional mAb (*B*). One representative of at least three similar experiments is shown; error bars show SD of triplicates of a representative experiment (*B*). *, $p < 0.05$ compared to no mAb. #, $p < 0.01$ compared to apoptosis via the HER-2/neu-directed mAb 520C9.

of the murine IgG1 Ab 520C9, apoptosis induction of SK-BR-3 cells was affected by the genetic polymorphism of FcγRIIa. Neutrophils isolated from FcγRIIa-R/R131 donors induced significantly higher levels of apoptosis than those isolated from FcγRIIa-H/H131 donors (Fig. 4). This FcγRIIa allotypic polymorphism has previously been shown to be relevant in several types of assays involving murine IgG1 Abs. These include anti-CD3-induced T

Table I. *Number of viable MDA-MB 453 nuclei after ADAC*[a]

	No mAb	HER-2/neu mAb	(FcαRI × HER-2)
$n \pm SD$[b]	12279 ± 451	5991 ± 297	6305 ± 461
%[c]	22 ± 1	13 ± 1	15 ± 2
p		0.001	0.0006

[a] After 20 h of coculture with PMN in the presence of the indicated Ab, staining with PI was performed as described in *Materials and Methods*. Her-2/neu mAb was 520C9 (murine IgG1), (FcαRI × HER-2) was generated from A77 and Herceptin, respectively. Assays were performed in quadruplicate; one of three similar experiments is shown. p for comparison with no mAb.

[b] The number ± SD of MDA-MB 453 nuclei with $G_0/_1$ or G_2 nuclear content is shown.

[c] The percentage ± SD of MDA-MB 453 nuclei with $G_0/_1$ or G_2 nuclear content is shown.

cell proliferation, phagocytosis of IgG-coated erythrocytes (37), and HER-2/neu Ab-dependent ^{51}Cr release with murine IgG1 Ab (4). Thus, the efficacy of murine IgG1 Abs in tumor therapy may also depend on the FcγRIIa allotype of the patients' effector cells. Interestingly, apoptosis was seen with E:T cell ratios as low as 10:1, which may reasonably occur in vivo. Targeting HER-2/neu, invasion of PMN into breast cancer metastases has been demonstrated in a phase I clinical trial (38).

However, the mechanism of apoptosis induction by neutrophils remains elusive. It is still unclear whether PMN start an intrinsic cell death program in the target cells, or whether, in analogy to the killing by cytotoxic T cells and NK cells, the release of the contents of neutrophils into the target cells is required. Recently, complement-mediated apoptosis of malignant B cell lines, opsonized with CD20-directed mAb, has been demonstrated in the absence of effector cells (39). Human neutrophils contain a rapidly mobilizable pool of so-called secretory vesicles, which are distinct from the azurophilic and specific granules. They contain plasma-derived proteins (40), e.g. albumin, and are referred to as "easily mobilizable vesicles." These secretory vesicles presumably also contain complement. Because PMN do not contain perforin, one may speculate that complement molecules released from secretory vesicles may permeabilize the cell membrane of target cells. This may enable the penetration of apoptosis-inducing enzymes into the cytosol of the target cells. PMN are continuously in a preapoptotic state (41). Therefore, apoptosis-inducing molecules, e.g., activated caspases, may be transferred into the target cell following cell surface contacts, and cytosolic communication after pore-forming or membrane fusion. Alternatively, an intrinsic cell death program, triggered by the engagement of cell surface molecules of the target cells, may operate.

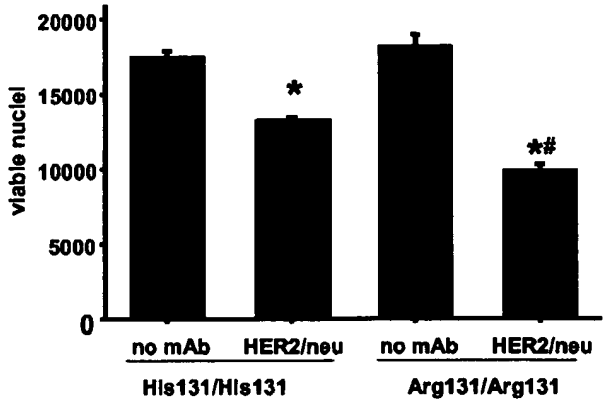

FIGURE 4. The R/H131 phenotype of the FcγRIIa on PMN effector cells influences apoptosis induction via murine IgG1 mAb 520C9 directed against HER-2/neu. The number of viable nuclei as measured by PI staining was significantly reduced in the presence of isolated PMN from donors homozygous for H131 or R131 of FcγRIIa on PMN (marked by *, denoting $p < 0.001$). However, the PMN from R131/R131 individuals were significantly more efficient in apoptosis induction than effector cells from H/H131 donors (marked by #, denoting $p < 0.001$). Data represent mean ± SEM of triplicates, one representative of three similar experiments is shown (30).

At high E:T cell ratios, as required for chromium release assays, cells are struck with such a strong impact that energy supply quickly ceases, repair function declines, and necrosis ensues. However, less impact, e.g., at low E:T cell ratios, is sufficient to initiate apoptosis. However, the execution of the energy-consuming death program takes time. We conclude that induction of apoptosis and necrosis both contribute to Ab-dependent killing by PMN of target cells.

In the context of biological tumor therapy, induction of tumor cell apoptosis seems to be profitable for the host. Nevertheless, nonopsonized apoptotic cells have been described to exert potent immunosuppressive effects (42). Mice immunized with apoptotic tumor cells mounted a significantly reduced humoral immune response when compared with animals injected with viable tumor cells (43). Therefore, the generation of huge amounts of apoptotic cells may suppress the immune response of the host, thereby enabling the immune escape of the tumors. However, in these experiments, apoptosis was induced by irradiation with UV B in nonimmunized animals, whereas in the present study, tumor cell apoptosis was induced by the concerted action of PMN and tumor-specific mAb via ADAC. Therefore, opsonization with Abs of tumor cells, may target the dying cells to FcR and complement-receptor mediated phagocytosis by professional APC, e.g., dendritic cells. Additionally, the activation of PMN during target cell apoptosis induction may herald a "danger signal" (44) for the immune system. In accordance with this hypothesis, PMN have been demonstrated to be critical in some animal models of tumor rejection (12). Surprisingly, depletion of T cells in a murine model of tumor therapy with FcγRI-directed bispecific Abs recognizing the idiotype of a B cell lymphoma resulted in an complete loss of efficacy (17). In conclusion, we demonstrated that PMN are able to kill tumor cell targets via Ab-dependent apoptosis induction. The exact mechanism of this apoptosis pathway and its role during tumor immune responses remain to be defined.

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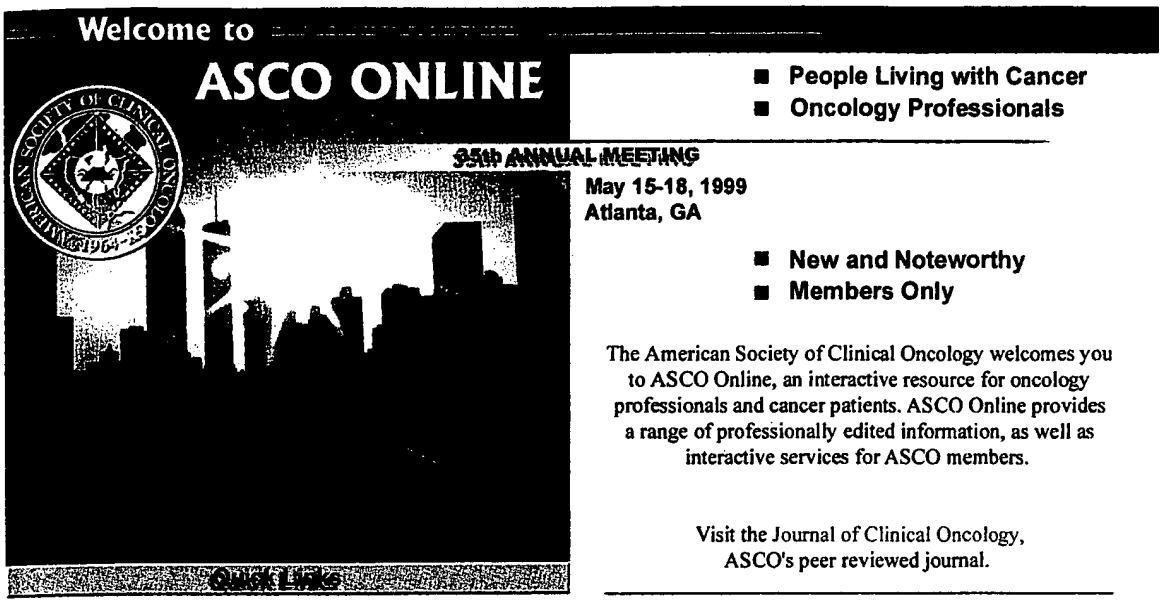

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